ISSN 0006-2979, Biochemistry (Moscow), 2021, Vol. 86, Suppl. 1, pp. S140-S151. © Pleiades Publishing, Ltd., 2021.
Russian Text © The Author(s), 2021, published in Uspekhi Biologicheskoi Khimii, 2021, Vol. 61, pp. 295-316.

REVIEW

Electroanalysis of Biomolecules: Rational Selection of Sensor Construction

Victoria V. Shumyantseva[1,2,a]*, Lubov E. Agafonova[1], Tatiana V. Bulko[1], Alexey V. Kuzikov[1,2], Rami A. Masamrekh[1,2], Jiayin Yuan[3], Dmitry V. Pergushov[4], and Larisa V. Sigolaeva[1,4]

[1]*Laboratory of Bioelectrochemistry, Orekhovich Research Institute of Biomedical Chemistry, 119992 Moscow, Russia*
[2]*Department of Biochemistry, Pirogov Russian National Research Medical University, 117997 Moscow, Russia*
[3]*Department of Materials and Environmental Chemistry, Stockholm University, 10691 Stockholm, Sweden*
[4]*Faculty of Chemistry, Lomonosov Moscow State University, 119991 Moscow, Russia*
[a]*e-mail: viktoria.shumyantseva@ibmc.msk.ru*

Received November 10, 2020
Revised November 18, 2020
Accepted November 18, 2020

Abstract—Methods of electrochemical analysis of biological objects based on the reaction of electro-oxidation/electroreduction of molecules are presented. Polymer nanocomposite materials that modify electrodes to increase sensitivity of electrochemical events on the surface of electrodes are described. Examples of applications electrochemical biosensors constructed with nanocomposite material for detection of biological molecules are presented, advantages and drawbacks of different applications are discussed.

DOI: 10.1134/S0006297921140108

Keywords: electroanalysis, multi-walled carbon nanotubes, biosensors, label-free detection, DNA, heme proteins

INTRODUCTION

Advances in modern biological chemistry involve, first and foremost, elucidation of fine details of metabolic pathways in pathological processes, identification of specific markers and their quantitative comparative evaluation for normal/pathological conditions. Various techniques and approaches to the analysis of biomolecules have been developed for diagnosis of diseases and for investigation of their interactions with molecular targets. Imaging techniques, atomic force microscopy, spectral methods, techniques using fluorogenic components, mass spectrometry of both macromolecules (e.g., proteomics) and low-molecular weight compounds (metabolomics) are being actively introduced to the modern translational medicine as representatives of nano- and omics technologies [1-4].

Abbreviations: DPV, differential pulse voltammetry; dsDNA, double-stranded DNA; LOD, limit of detection; Mb, myoglobin; MWCNT, multi-walled carbon nanotubes; PILs, poly(ionic liquid)s; SPE, screen-printed graphite electrode; Trp, tryptophan; Tyr, tyrosine.

* To whom correspondence should be addressed.

Electrochemical methods in biochemical and biomedical studies are actively developing as a result of productive combination of nanotechnologies, nanomaterials, and methods for recording and processing of the results of electroanalysis [5]. The advantage of electrochemical methods is quantitative determination of electroactive biocomponents based on the fundamental laws of electrochemistry; moreover, measuring devices can be both stationary, for conducting fundamental studies, and portable, for clinical tests. By recording the dependence of the current flow through an indicator electrode in contact with an electroactive substance on the potential difference between the working and reference electrodes, it is possible to obtain data on concentration of the electroactive substance, kinetics and thermodynamics of electrochemical reaction [6]. Type of the electrode and its modification for immobilization of biomolecules play crucial roles in efficiency of electrochemical process associated with electron transport and/or electrocatalysis. Unmodified electrodes are not always sufficiently effective. Different materials can be used as modifiers: polymers, gels, native biomolecules, metals and their oxides. Nanosized structures have become widespread in recent

years, including colloidal solutions of gold and silver (metal nanoparticles), iron oxides, single- and multi-walled carbon nanotubes, graphene, graphene oxide, boron- nitrogen-, and sulfur-doped graphene, lipids, synthetic membrane-like materials, and polymeric compositions [6-13]. Nanoparticles can catalyze reactions occurring at the surface of the electrode. Surface modification of the electrodes with nanoparticles (nanostructuring) allows selection of optimal conditions and tuning the sensor performance according to the required electrochemical reaction or analyzed substance, as well as provides necessary analytical characteristics of the method such as biocompatibility, appropriate limit of detection, selectivity, range of the analyte concentrations, etc.

The type of modification of the working surface of electrode is often selected empirically by examining combinations and concentrations of the components. In the present review, we describe the approaches to various types of modifications based primarily on the properties of biomolecules, as well as properties of modifiers as direct participants and/or mediators between the redox-center of the biomolecule and the electrode. The methods of electroanalysis of functionally significant hemoproteins such as myoglobin, involved in the transport of oxygen in muscles, and cytochrome *c*, involved in electron transport, as well as of oligonucleotides and double-stranded DNA (dsDNA) are reviewed.

Hemoproteins play a key role in biochemical processes such as transport and storage of molecular oxygen by hemoglobin and myoglobin, electron transport from respiratory substrates by cytochromes and terminal oxidation of O_2 by cytochrome *c* oxidase, decomposition of hydrogen peroxide by catalase, oxidation of organic compounds by peroxidase, metabolic transformation of drugs and xenobiotics in phase I reactions by cytochrome P450 enzymes, nitric oxide synthesis from L-Arg by NO synthase, and use of reactive oxygen species by catalase. These various functions are based primarily on the redox properties of the iron ion of heme according to the $Fe(III) + e \leftrightarrow Fe(II)$ scheme [14, 15]. The effect of direct electron transfer from the electrode to the heme iron is widely used for designing electrochemical hydrogen peroxide and nitrite ion sensors.

Cardiac myoglobin and cytochrome *c* are the markers of acute myocardial infarction [15-17]. Cytochrome *c* is an important heme-containing metalloprotein localized in the cytosol between the inner and outer mitochondrial membranes. Cytochrome *c* belongs to the class I *c*-type cytochrome family and performs different functions depending on its cellular localization and conditions. It mediates electron transfer between respiratory chain complexes III and IV [18, 19]. In addition, the release of cytochrome *c* from mitochondria is a signal for initiating of apoptotic process. Detection of cytochrome *c* in the extracellular space can be used in clinical diagnostics of pathologies such as acute myocardial infarction, lupus erythematosus, rheumatoid arthritis, oncologic diseases. Cytochrome *c* is used as a biomarker to identify mitochondrial damage leading to cell death. Proapoptotic properties of cytochrome *c* can be used for the development of diagnostic techniques, in search for new drugs capable of killing pathological cells, and for assessment of efficiency of the drugs. The development of modern, rapid and affordable methods for quantitative determination of cytochrome *c* with electrochemical biosensors is an urgent task of bioanalytical chemistry [20].

Nucleotides, oligonucleotides, DNA and RNA are considered the markers of many pathological states. The presence of mutations in the circulating tumor DNA, as well as in RNA or microRNA, and their amounts can be diagnostic biomarkers of various oncologic diseases and prognostic markers for the analysis of response to treatment and/or as markers of disease progression [21-29]. The change in DNA length (DNA fragmentation) is one of the recognized markers of programmed cell death (apoptosis) [24]. Analysis of the modified heterocyclic bases (e.g., methylation profiles) is crucial both for epigenetic studies [24, 30-33] and for detection of point mutations [30].

Thus, the development of highly sensitive electrochemical sensors for DNA and functionally significant hemoproteins is a relevant issue. The present work describes screen-printed graphite electrodes (SPE) modified with polymeric nanocomposites based on multi-walled carbon nanotubes (MWCNT) to amplify electrochemical signals of biochemical processes on the electrode surface and to improve limit of detection in electroanalysis of different biological objects: oligonucleotides, double-stranded DNA, myoglobin, and cytochrome *c*.

Polymeric materials are able to disperse carbon materials and form stable colloidal dispersions of carbon nanoparticles (MWCNT) in aqueous solutions. In addition, polymers may contain charged and hydrophobic segments of different length. Such property of the polymers makes them attractive as electrode modifiers for electrochemical detection of biological objects, which also have both hydrophobic domains and polar regions enriched with positive or negative charges. In addition, graphite electrodes that are widely used in electroanalysis have both hydrophobic component and negatively charged carboxylic groups emerging due to oxidation of carbon atoms. This combined set of properties of polymers, biological objects, and surface of indicator electrodes make this triad interconnected and show the possibility of controlling efficiency of the entire system.

ELECTROANALYSIS OF MYOGLOBIN USING POLYMER NANOCOMPOSITES FOR ELECTRODE MODIFICATION

Myoglobin (Mb) is a hemoprotein (MW 16,900 Da); its main function is oxygen transport in muscles.

Myoglobin is a molecular marker in pathogenesis of cardiovascular diseases and an early indicator of myocardial infarction [16]. Most researchers consider normal myoglobin concentration to be 100 μg/liter. Isoelectric point of myoglobin is pI = 7; at physiological pH levels, this protein is uncharged. Myoglobin is an electroactive protein; the methods of its detection are based on the direct registration of the signal of heme iron reduction [Mb-Fe(III) + e^- ↔ Mb-Fe(II)].

The reduced form of myoglobin, Mb-Fe(II), actively binds oxygen according to the Mb-Fe(II) + O_2 → → [Mb-Fe(II)O_2] scheme; thus, under aerobic conditions, it is possible to record only the reduction peak of this hemoprotein, which reflects the process of electrocatalytic reduction and amplifies the signal recorded from this protein. Due to the functional significance of myoglobin, various modifiers of the electrode surface have been developed: surfactants, polymeric hydrogels,

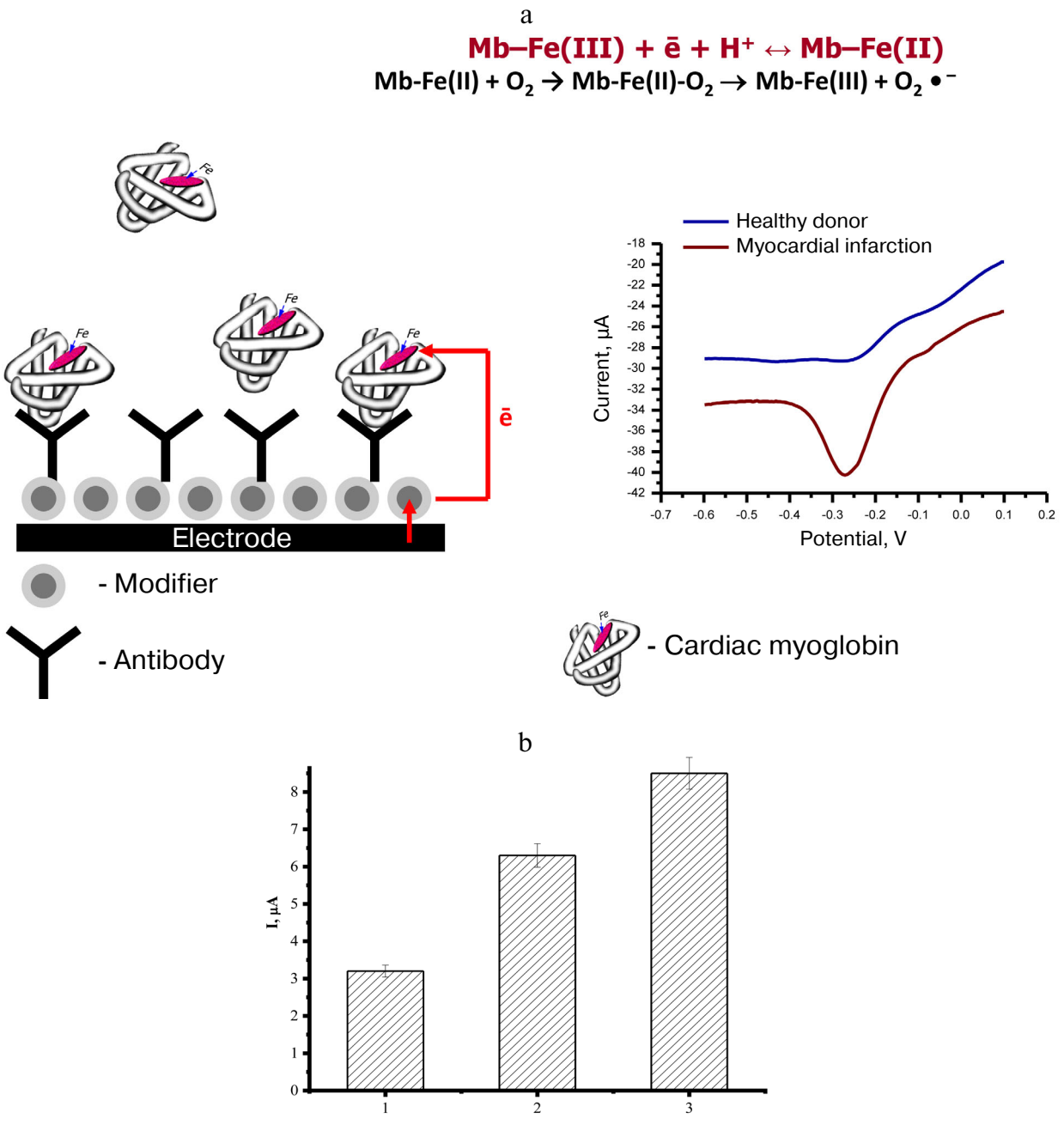

Fig. 1. a) Scheme of electroanalysis of cardiac myoglobin using modified electrodes with immobilized antibodies. b) Dependence of the maximum amplitude of the current of differential pulse voltammetry (DPV) SPE/MWCNT/Mb (1), SPE/MWCNT/PB_{290}-*b*-$PDMAEMA_{240}$ (0.5 mg/ml)/Mb (2), SPE/MWCNT/PB_{290}-*b*-$PDMAEMA_{240}$ (2.0 mg/ml)/Mb (3) in aerobic 100 mM potassium phosphate buffer, 50 mM NaCl, pH 7.4. DPV conditions: scanning speed, 50 mV/s; amplitude, 20 mV; increment, 5 mV; frequency, 10 Hz. (Colored versions of Figs. 1-6 are available in online version of the article and can be accessed at: https://www.springer.com/journal/10541)

membrane-like polyelectrolyte/surfactant complexes, aluminosilicates, silk fibroin, lipids, carbon nanotubes, ionic liquids, and metal oxides [34]. Surfactants and polymers (sodium dodecyl sulfate, chitosan) are also used to improve dispersibility of MWCNT and to increase colloidal stability of the dispersions of carbon nanomaterials [35]. Efficiency of the direct electron transfer between the electrode and the heme iron ion depends on the used electrode material, modification of the electrode surface, and proper orientation of the protein active site on the electrode. Composite nanomaterials improve electron transfer, contribute to fixation of biological material on the electrode surface, and provide biocompatibility to preserve natural affinity and catalytic properties of biomolecules. Modification of electrodes by MWCNT imparts some useful properties to the sensors, such as increased surface area, improved conductivity, and broad range of working potentials [32, 36].

Previously we observed the synergistic effect of MWCNT in combination with the ionic amphiphilic diblock copolymer, polycationic poly(1,2-butadiene)-*block*-poly(*N*,*N*-dimethylaminoethyl methacrylate) (PB_{290}-*b*-$PDMAEMA_{240}$) in electrochemical detection of myoglobin [35]. It was demonstrated that the polymer PB_{290}-*b*-$PDMAEMA_{240}$ is an effective biocompatible material for incorporation of Mb, which facilitated direct electron transfer from the electrode to the hemoprotein. Binding specificity was provided by the antibodies to cardiac myoglobin immobilized on the electrode (Fig. 1a). The polymer forms micelles in aqueous solutions which show good adhesion to carbon materials at pH = 7 in phosphate buffer and they form thin films on the hydrophobic graphite substrate. This type of polycationic polymer can form ionic bonds with the negatively charged groups of myoglobin molecules and interact with carboxylic groups of the working surface of the graphite electrode, which are formed due to partial oxidation of carbon atoms. An almost 3-fold increase in Mb reduction current was achieved in the MWCNT (2 mg/ml)/ PB_{290}-*b*-$PDMAEMA_{240}$ matrix compared to the electrode modified only by MWCNT dispersed in chloroform (Fig. 1b).

The layer-by-layer deposition of modifiers onto the surface of the working electrode was used in this work: layer 1, MWCNT dispersion in chloroform; layer 2, polycationic polymer PB_{290}-*b*-$PDMAEMA_{240}$. In addition, the role of MWCNT should be mentioned: the amplitude of DPV cathode current increases 1.4-fold with the increase in MWCNT concentration from 0.5 mg/ml to 2 mg/ml. Sensitivity of this biosensor system is sufficient to cover the whole range of Mb concentrations, from the normal physiological concentration of human cardiac myoglobin (10-100 ng/ml; 0.56-5.6 nM) to the Mb level in patients with myocardial infarction (100-1780 ng/ml; 5.6-100 nM) [37].

MODIFICATION OF ELECTRODES BY MWCNT DISPERSIONS IN AQUEOUS SOLUTIONS OF POLY(IONIC LIQID)s AND CATIONIC DIBLOCK COPOLYMERS FOR DNA ANALYSIS

Nucleotides, oligonucleotides, DNA, microRNA, and RNA are known as markers of various pathological conditions. The donor-specific DNA circulating in transplant recipients was suggested as a potential biomarker of organ rejection or graft injury [28]. The adenine and guanine levels in plasma, serum and urine, as well as the change of adenine concentration in DNA, could be considered as an indicator of carcinoma or liver diseases [29]. Analysis of the circulating tumor DNA in blood plasma is considered as a diagnostic and prognostic method in oncology referred to as "liquid biopsy" [25, 38].

The methods of electroanalysis based on electrochemical reaction of electro-oxidation of heterocyclic bases have been developed for quantitative determination of DNA, RNA, nucleotides, and oligonucleotides [39-41].

The pyrimidine heterocyclic bases (thymine and cytosine) are electro-oxidized at substantially higher potentials (more than 1.2-1.4 V), which complicates their recording with graphite electrodes obtained by screen-printing. Purine heterocyclic bases (adenine and guanine) are electrochemically oxidized at lower potentials (0.6-0.9 V). Various approaches have been developed for analysis of purine heterocyclic bases in DNA, RNA, nucleotides, and oligonucleotides [24, 33, 42-52].

Electro-oxidation of guanine is an irreversible process involving transfer of two protons and two electrons with the formation of 8-oxoguanine. This compound serves as a biomarker of DNA damage and fragmentation due to oxidative stress [31, 46]. The mechanism of adenine electro-oxidation is also irreversible and includes three stages that involve two electrons and formation of 2-oxoadenine in the first stage, followed by two stages involving four additional electrons and formation of 2,8-dioxoadenine and its oxidized form [31, 46] (Fig. 2).

In our studies, we took advantage of the properties of polycationic polymers to disperse carbon nanomaterials in aqueous media and to interact with polyanionic molecules of DNA [53, 54].

The uniform coating of working electrode requires a highly homogenous MWCNT dispersion with high conductivity and without (or, at least, with the minimum) structural damage to nanomaterial. However, it is rather difficult to produce such MWCNT dispersion due to the low dispersibility of such carbon nanomaterials in the most of organic solvents. Highly destructive functionalization of MWCNT by hard oxidation in the concentrated mixture of nitric and sulfuric acids is currently replaced by the safer and milder ultrasonic treatment either in water or in aqueous-organic mixtures and organic solvents

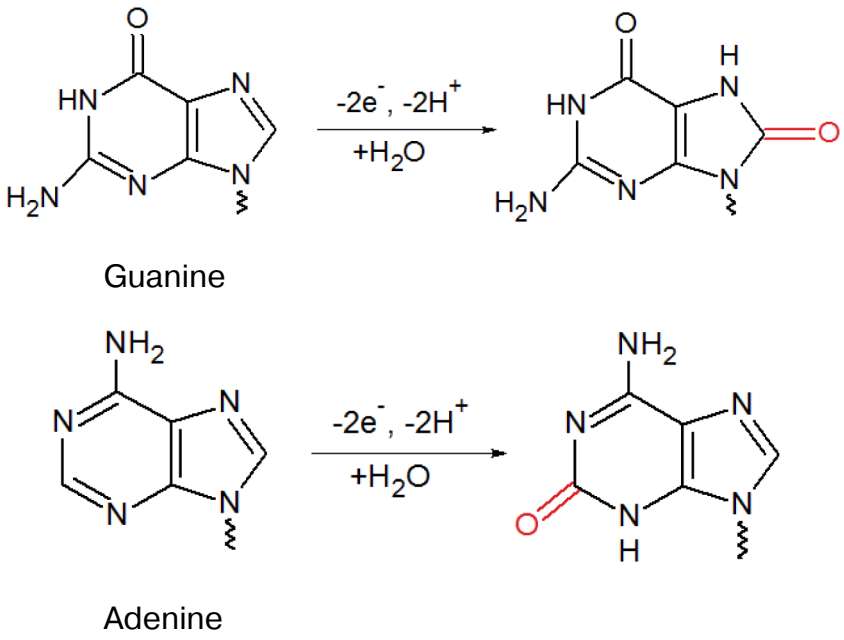

Fig. 2. Scheme of electrochemical oxidation of guanine and adenine [31, 46].

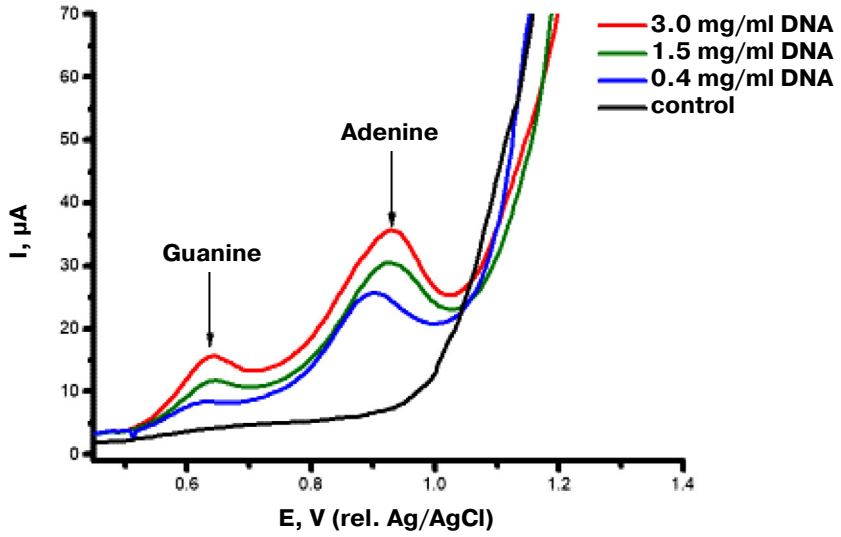

Fig. 3. Differential pulse voltammograms of the electrodes modified by MWCNT/PIL-Et; DNA concentration, 0.4-3.0 mg/ml.

(dimethylformamide, acetone, isopropanol, ethanol, toluene, N-methyl-2-pyrrolidone, cyclodextrins).

Polymeric materials carrying various functional groups can be used for immobilization of biological objects that differ in the molecular charge and isoelectric points. The polyanionic molecules of dsDNA were immobilized using polycationic polymeric modifiers such as poly(ionic liquid)s based on imidazole derivatives [53]. For direct electroanalysis of dsDNA based on electrooxidation of guanine and adenine, poly(ionic liquid)s (PIL) on the basis of imidazole derivatives, poly(1-ethyl-3-vinylimidazolium bromide, PIL-Et) and poly(1-butyl-3-vinylimidazolium bromide, PIL-But) were synthesized and used for modification of SPE surface by highly stable MWCNT dispersions in aqueous media [53]. Figure 3 shows the DVP signals from electrodes modified by

MWCNT/PIL-Et with DNA concentration varying within a range of 0.4-3.00 mg/ml [53].

Such SPE modification considerably increases the electroactive surface area and the rate of electron transfer due to synergistic combination of specific properties of MWCNT, such as strong adsorption, high electron-transporting capacity and high specific surface area, and advantages of the PIL such as ionic conductivity and ability to disperse carbon materials. In addition, the positively charged nitrogen atom of the imidazole ring of polymeric constructs based on PIL can form ionic bonds with the negatively charged phosphate groups of oligonucleotides or DNA, stabilizing the system for recording and measuring of electrochemical signals.

The synthesis of PIL for preparation of stable MWCNT dispersions is described in [53]. Analytical characteristics of the sensors based on recording electro-oxidation of guanine (with the potential E = +0.60 ± 0.01 V) and adenine (with the potential E = +0.85 ± 0.01 V) are presented in Table. Linear ranges of dsDNA determination correspond to 5-500 μg/ml for the oxidation peak of guanine and 0.5-50 μg/ml for the oxidation peak of adenine (table).

Since the maximum amplitude of oxidation peaks of purine nucleotides is proportional to the content of adenine (A) or guanine (G) residues in the nucleotide chain, direct electro-oxidation can be used to detect point mutations or the so-called single-nucleotide polymorphism (SNP) in short DNA fragments (oligonucleotides). The pair of oligonucleotides differing in only one nucleotide residue was used as an example demonstrating ability of the assay to detect SNPs.

The developed SPE/PIL-But/MWCNT systems were able to recognize point mutation in 12-membered single-stranded oligonucleotides (Fig. 4): 5′-AAA-CCC-G**A**C-CGG-3′ and 5′-AAA-CCC-G**T**C-CGG-3′ [53, 54]. The oligonucleotide sequence and position of the mutation were chosen as analogs of the fragment of epidermal growth factor receptor (EGFR) 21. Figure 4 clearly shows the difference between the values of current of adenine electro-oxidation I_A for these oligonucleotides: the oxidation current for A-residues was about 6.7 μA and 3 μA for oligonucleotide 1 and 2, respectively, due to different contents of A-residues, while the oxidation current for guanine was the same for both oligonucleotides, indicating that there was no difference in the content of G-residues. Thus, this experiment demonstrated potential of the developed SPE/PIL-But/MWCNT-based sensor to detect point mutations in short DNA fragments.

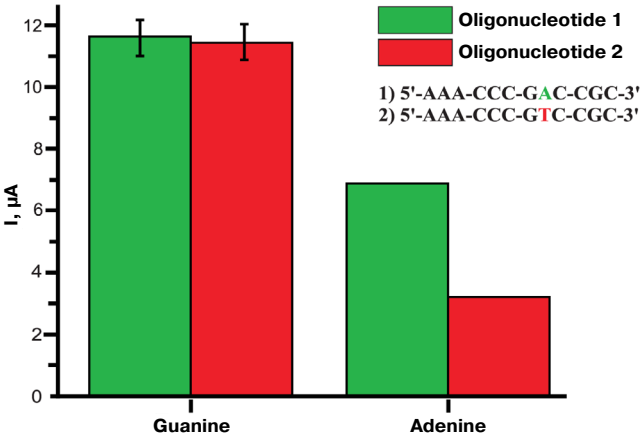

Fig. 4. Electroanalysis of oligonucleotides (1) 5′-AAA-CCC-G**A**C-CGG-3′ and (2): 5′-AAA-CCC-G**T**C-CGG-3′ using nanostructured electrodes SPE/PIL-But/MWCNT.

Non-small cell lung carcinoma is associated with exon 21 L858R mutation in the EGFR and, as is well known, this is a disease with poor prognosis [30]. The exon 21 L858R mutation is of great clinical significance for choosing treatment strategy. Detection of such mutations is very important from the clinical point of view and could be part of personalized treatment.

A method was developed to produce homogenous and stable colloidal dispersion of MWCNT in aqueous solution of ionic amphiphilic diblock copolymer, polycationic poly(1,2-butadiene)-*block*-poly(*N*,*N*-dimethylaminoethyl methacrylate) (PB_{290}-*b*-$PDMAEMA_{240}$) [55] and diblock copolymers P*n*BMA_x-*b*-$PDMAEMA_y$ poly(*n*-butylmethacrylate)$_x$-*block*-poly(*N*,*N*-dimethylaminoethyl methacrylate)$_y$, where the x and y are varied from (x = 40, y = 40) to (x = 40, y = 120) and (x = 70, y = 120) [56]. The 5 mg/ml aqueous solutions of diblock

Analytical characteristics of chemically modified electrodes for the analysis of biological objects

Biological object	Type of electrode, modification	Working concentrations range
dsDNA	SPE/PIL/MWCNT	5-500 μg/ml [guanine peak (G)], E = +0.60 ± 0.01 V 0.5-50 μg/ml [adenine peak (A)], E = +0.85 ± 0.01 V
dsDNA	SPE/P*n*BMA_{40}-*b*-$PDMAEMA_{120}$/MWCNT	5-1500 μg/ml [guanine peak (G)], LOD = 5 μg/ml and 1-200 μg/ml [adenine peak (A)], LOD = 1 μg/ml
Cytochrome *c*	SPE/P*n*BA_{100}-*b*-PAA_{140}/MWCNT	1-100 μM, LOD = 1.16 μM at E = +0.578 ± 0.011 V (Tyr + Trp)
Mb	SPE/PB_{290}-*b*-$PDMAEMA_{240}$/MWCNT	physiological concentrations 10-1780 ng/ml (0.56-100 nM)

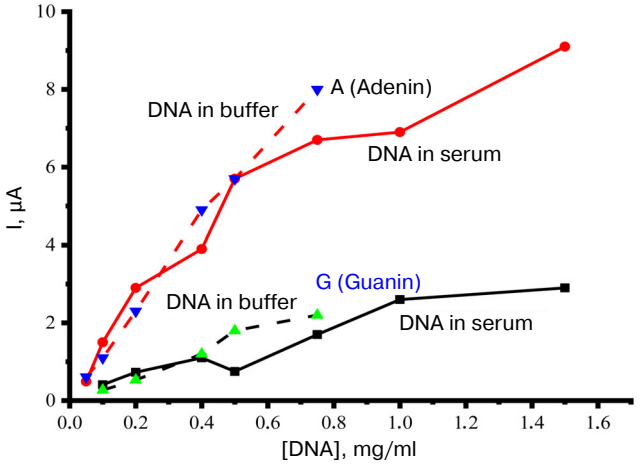

Fig. 5. Comparison of dsDNA determination in buffer and in serum by analyzing electrochemical oxidation of guanine and adenine using the constructed P*n*BMA$_{40}$-*b*-PDMAEMA$_{120}$/MWCNT (2 mg/ml MWCNT) sensor.

copolymers P*n*BMA$_x$-*b*-PDMAEMA$_y$ were prepared by direct dissolution; all DMAEMA monomeric units of diblock copolymers were protonated at pH 3.

The authors of the work [56] reported the use of amphiphilic ionic poly(*n*-butylmethacrylate)-*block*-poly(*N*,*N*-dimethylaminoethyl methacrylate) diblock copolymers (P*n*BMA$_{40}$-*b*-PDMAEMA$_{40}$, P*n*BMA$_{40}$-*b*-PDMAEMA$_{120}$, P*n*BMA$_{70}$-*b*-PDMAEMA$_{120}$) containing both hydrophobic and ionic blocks in one macromolecule for dsDNA electroanalysis. In addition to the fact that these diblock copolymers are polymer binders facilitating sufficient integrity of the deposited layer of MWCNT during modification of the electrode, P*n*BMA$_x$-*b*-PDMAEMA$_y$ themselves can also act as a host matrix. Their protonated PDMAEMA efficiently retain the oppositely charged target analytes (in this particular case, the negatively charged dsDNA), thereby providing potential advantages for electrochemical measurements. The maximum combined effect was shown for the SPE/P*n*BMA$_{40}$-*b*-PDMAEMA$_{120}$/MWCNT system manufactured using the dispersed MWCNT at a concentration of 2 mg/ml.

Optimal ratio of the hydrophobic and hydrophilic fragments of the diblock copolymer PnBMA$_{40}$-*b*-PDMAEMA$_{120}$ and the MWCNT concentration of 2 mg/ml results in substantial increase in the electroactive surface area both due to the particular features of the copolymer structure (optimal hydrophobic–hydrophilic balance and optimal total length of the macromolecule) and due to the optimal content of carbon nanotubes in the P*n*BMA$_x$-*b*-PDMAEMA$_y$/MWCNT system. The linear ranges of dsDNA detection with this system correspond to 5-1500 μg/ml for guanine and 1-200 μg/ml for adenine (table; Fig. 5). The developed sensors allow DNA determination in human blood serum.

The limits of detection (LOD) are 5 and 1 μg/ml for guanine and adenine, respectively. Based on the average molecular weight of dsDNA from sturgeon milt of 20 kDa, the molar values of the limits of detection are 0.25 μM for guanine and 0.05 μM for adenine. It was shown [56] that the developed SPE/P*n*BMA$_{40}$-*b*-PDMAEMA$_{70}$/MWCNT

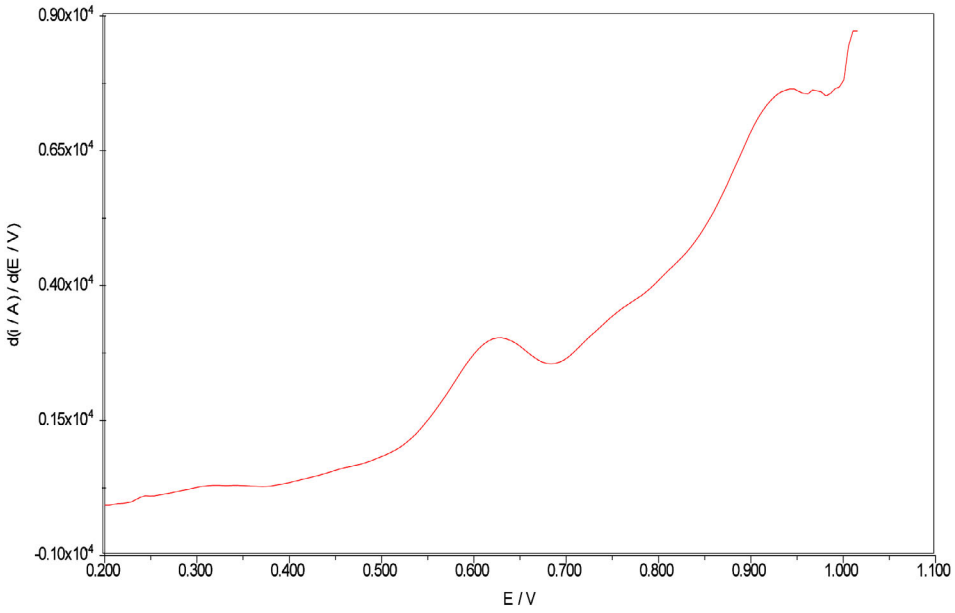

Fig. 6. Intensity of the peaks of differential pulse voltammetry during electrochemical oxidation of guanine and adenine of the leukocyte DNA at concentration of 9.35 μg/ml analyzed with the SPE/P*n*BMA$_{40}$-*b*-PDMAEMA$_{120}$/MWCNT (2 mg/ml MWCNT) system. Experimental data were obtained by signal processing with First Derivative Test by differentiating voltammograms.

system could be used for the analysis of dsDNA isolated from human blood leukocytes in the range of concentrations 2-100 μg/ml (Fig. 6).

Polycationic polymers used for construction of sensors for analysis of polyanionic DNA molecules allow efficient DNA detection by analyzing the signals of electro-oxidation of the purine bases (guanine and adenine) within a broad and physiologically significant range of concentrations.

MODIFICATION OF ELECTRODES BY MWCNT DISPERSIONS IN AQUEOUS SOLUTIONS OF ANIONIC DIBLOCK COPOLYMERS FOR ANALYSIS OF CYTOCHROME *c*

Cytochrome *c* (MW 13,370 Da) is of great clinical significance as a marker of myocardial infarction, a marker of apoptosis, and an electron transport hemoprotein. The following methods have been developed for quantification of cytochrome *c*: immunochemical and spectral methods, methods based on recording radiation emitted by the product of reaction between the analyzed substance and chemiluminescence enhancer, and electrochemical methods [19, 57].

The iron ion of the heme is reversibly transformed from the oxidized form (Fe^{3+}) into the reduced form (Fe^{2+}) during electron transfer, which leads to the changes in the heme conformation. Redox potential of this process determines efficiency of the electron transfer. Even minor changes in the structure of polypeptide chain and microenvironment of the heme can affect efficiency of the electron transfer and functioning of the entire respiratory system and apoptotic processes. Isoelectric point of cytochrome *c* is pI = 10.1; it implies predominance of the positively charged side-chain radicals in the molecule at neutral pH, and that cytochrome *c* is positively charged under physiological conditions. In the mitochondrial membrane, about 15% of cytochrome *c* is tightly bound to cardiolipin, one of the phospholipids forming this membrane. The complex of cytochrome *c* with cardiolipin exhibits peroxidase activity, initiating cell apoptosis. Cardiolipin contains mostly side-chain radicals with anionic groups. Cardiolipin comprises about 15-20% of all lipids of the inner mitochondrial membrane and has a unique structure consisting of four fatty acids, with the maximum content of linoleic acid. Due to the positive charge of cytochrome *c* in the neutral medium, it binds through electrostatic interactions to the surface of the negatively charged membrane lipid bilayer [19, 58].

Taking into account this property of cytochrome *c*, the composite materials for modification of the electrodes have been developed that carrying negative charges for stabilization and binding of cytochrome *c*.

Cytochrome *c* was immobilized on the surface of the electrode using MWCNT dispersions based on poly(*n*-butylacrylate)-*block*-poly(acrylic acid) P*n*BA_{100}-*b*-PAA_{140} [59].

This type of electrode modification provides possibility for generation of panoramic voltamperogram in a broad range of potentials and allows recording not only redox processes of the heme iron ion (according to the reaction Fe(III) + e↔Fe(II)) at near-zero potential, but also studying the processes of electrochemical oxidation of amino acids of the polypeptide chain of cytochrome *c* (tyrosine and tryptophan at +0.6 V), as well as irreversible electrochemical oxidation of the heme at +0.8 V (Fig. 7).

Such multipoint detection can be considered as an electrochemical fingerprint of cytochrome *c* providing a method for recognition and quantitative determination of cytochrome *c* in complex (bio)chemical analytes. The limit of detection of cytochrome *c* at E = +0.578 ± 0.011 V related to the Tyr + Trp oxidation was 1.16 μM, i.e., approximately twice lower than for the method based on reduction of the heme at near-zero potential. The study of the processes of electro-oxidation of amino acids of the cytochrome *c* polypeptide chain (tyrosine and tryptophan) is important also from the point of recording functionally significant posttranslational modifications of this hemoprotein [60]. Tyrosine is subject to posttranslational modification such as phosphorylation and nitration. The human cytochrome *c* contains five tyrosine residues. Tyr46 and Tyr48 are localized in the Ω loop positioned close to the heme. Nitration of these residues causes change in the secondary structure due to redistribution of the network of hydrogen bonds surrounding the heme. As a result, the heme is converted into the high-spin state, which leads to increase in the peroxidase activity of nitrated cytochrome *c* and induces specific degradation of the protein. Nitration of the Tyr46 and Tyr48 residues in cytochrome *c* leads to the assembly of nonfunctional apoptosome. Furthermore, nitration of the Tyr67 residue does not affect secondary structure of this hemoprotein but nitration of the Tyr74 residue results in the increase of peroxidase activity, impairment of interaction with caspase 9, and inhibition of apoptosis. Nitration of the Tyr46, Tyr48, Tyr67, Tyr74, and Tyr97 residues changes redox potential of cytochrome *c* and disrupts its interactions with caspases, which in turn results in the assembly of nonfunctional defective apoptosome. Nitration of the Tyr74 residue is accompanied by the sevenfold increase in the peroxidase activity of cytochrome *c*, as well as the loss of electron-transport functions due to the shift of the redox potential to 400 mV.

Tyr97 is the key amino acid residue in the formation of apoptosome [61, 62]. Tyr97 phosphorylation leads to formation of a salt bridge between the phosphotyrosine and Lys7, which interferes with apoptosome formation [63, 64].

Thus, a strategy has been developed to obtain an electrochemical signature of cytochrome *c* heme protein,

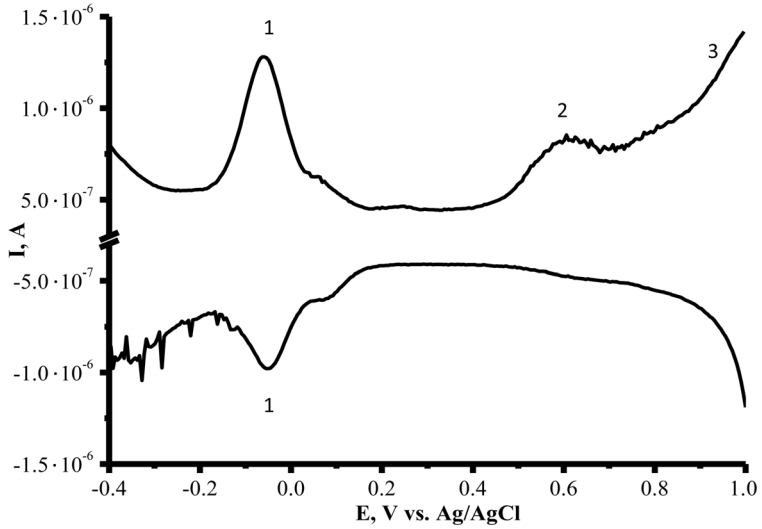

Fig. 7. Panoramic DPV of cytochrome *c* on SPE/MWCNT/P*n*BA$_{100}$-*b*-PAA$_{140}$ as an electrochemical fingerprint of cytochrome *c*: (1) heme area, (2) oxidation of amino acids tyrosine and tryptophan Tyr + Trp, (3) electro-oxidative destruction of the heme.

based on both electroanalysis of the prosthetic group of the protein and electro-oxidation properties of the amino acid residues such as Tyr and Trp. The LOD was 1.91 μM in the case of recording the heme signal and 1.16 μM in the case of recording electro-oxidation. The specific and sensitive signature of cytochrome *c* can be further used for monitoring apoptotic events during chemotherapy, searching for novel antitumor drugs, studying mechanism of apoptosis, identifying posttranslational modifications of particular functionally significant amino acid residues of the polypeptide chain or of the heme itself.

CONCLUSIONS

Selection of the appropriate modification of electrode for immobilization of biological objects with different properties and for recording electron transport plays an important role in the development of efficient electrochemical process associated with electron transfer and electrocatalysis. Exploiting of MWCNT provides electrodes with increased surface area, improved conductivity, and broad potential window. Moreover, combination of MWCNT and polymers not only facilitates production of highly homogenous dispersion of carbon nanomaterials but also enhances the synergistic effect and allows effective investigation of biological objects with differently charged molecules and different isoelectric points.

Modifications of the screen-printed graphite electrodes with sensor constructs based on the dispersions of MWCNT in aqueous solutions of polymers are very promising for the development of highly sensitive recognition systems for quantitative analysis of biological objects with different molecular parameters (charge, hydrophobicity, size, functions). Carbon nanomaterials used for modification of electrodes increase analytical sensitivity of the system and amount of the electroactive component on the electrode, while polymers not only facilitate preparation of highly homogenous MWCNT dispersion with high conductivity but also mediate tight attachment of the analyzed biological objects on the working surface of the electrode (table).

The evidence-based selection and investigation of the mechanisms of specific interactions (ionic, hydrophobic) of a biomolecule and an electrode modifier are being studied actively both theoretically and experimentally. The authors of the work [65] used the approaches and methods of molecular dynamics to understand the mechanism of adsorption of alanine, glycine and valine on the surface of graphene and functionalized graphene, which is actively used for modification of electrodes in order to improve analytical characteristics of the sensors. This work is an important step in understanding principles for selections of biomolecule/modifier tandem that have predictive power.

Functionalization of the electrode surface plays an important role in the development of sensor systems for biochemistry, clinical medicine, and pharmacology. Based on the goal of the experiment (analysis of the marker of pathological process, search for potential substrates/inhibitors of the functionally significant enzymes, study of drug interactions, analysis of conformational changes in proteins, the search for single nucleotide substitutions) and the selected electrochemical method of analysis, it is possible to increase sensitivity of the electrochemical biosensor system and to decrease the limit of quantification, which is essential for constructing "smart" biosensors for electroanalysis of various biological objects.

Funding. The work was done in the framework of the Russian Federation fundamental research program for the long-term period for 2021-2030. The modification of electrode surfaces by polymer materials was financially supported by the Russian Science Foundation (project no. 18-44-04011).

Ethics declarations. The authors declare no conflict of interest in financial or any other sphere. This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES

1. Bogdanov, A. A., Jr., Solovyev, I. D., and Savitsky, A. P. (2019) Sensors for visualization of proteolytic activity and their applications in human disease models, *Usp. Biol. Khim.*, **59**, 3-38.
2. Lokhov, P. G., Balashova, E. E., Trifonova, O. P., Maslov, D. L., and Archakov, A. I. (2020) Ten years of Russian metabolomics: history of development and basic results, *Biomed. Khim.*, **66**, 279-293.
3. Trifonova, O. P., Balashova, E. E., Maslov, D. L., Grigoryev, A. I., Lisitsa, A. V., et al. (2020) Metabolomic analysis of blood for creation of a digital image of healthy person, *Biomed. Khim.*, **66**, 216-223.
4. McShane, L. M., Cavenagh, M. M., Lively, T. G., Eberhard, D. A., Bigbee, W. L., et al. (2013) Criteria for the use of omics-based predictors in clinical trials, *Nature*, **502**, 317-320.
5. Shumyantseva, V. V., Bulko, T. V., Sigolaeva, L. V., Kuzikov, A. V., Pogodin, P. V., and Archakov, A. I. (2018) Molecular imprinting coupled with electrochemical analysis for plasma samples classification in acute myocardial infarction diagnostic, *Biosens. Bioelectron.*, **99**, 216-222.
6. Shumyantseva, V. V., Kuzikov, A. V., Masamrekh, R. A., Bulko, T. V., and Archakov, A. I. (2018) From electrochemistry to enzyme kinetics of cytochrome P450, *Biosens. Bioelectron.*, **121**, 192-204.
7. Mi, L., He, F., Jiang, L., Shangguan, L., Zhang, X., et al. (2017) Electrochemically-driven benzo[*a*]pyrene metabolism via human cytochrome P450 1A1 with reductase coated nitrogen-doped graphene nano-composites, *J. Electroanal. Chem.*, **804**, 23-28.
8. Sharma, S., Singh, N., Tomar, V., and Chandra, R. (2018) A review on electrochemical detection of serotonin based on surface modified electrodes, *Biosens. Bioelectron.*, **107**, 76-93.
9. Anzar, N., Hasan, R., Tyagi, M., Yadav, N., and Narang, J. (2020) Carbon nanotube – a review on synthesis, properties and plethora of applications in the field of biomedical science, *Sens. Int.*, **1**, 100003.
10. Carrara, S., Baj-Rossi, C., Boero, C., and De Micheli, G. (2014) Do carbon nanotubes contribute to electrochemical biosensing? *Electrochim. Acta*, **128**, 102-112.
11. Hu, C., and Hu, S. (2009) Carbon nanotube-based electrochemical sensors: principles and applications in biomedical systems, *J. Sensors*, **2009**, 1-40.
12. Baig, N., Sajid, M., and Saleh, T. A. (2019) Recent trends in nanomaterial-modified electrodes for electroanalytical applications, *Trends Anal. Chem.*, **111**, 47-61.
13. Rivera-Gavidia, L. M., Luis-Sunga, M., Bousa, M., Vales, V., Kalbac, M., et al. (2020) S- and N-doped graphene-based catalysts for the oxygen evolution reaction, *Electrochim. Acta*, **340**, 135975.
14. Shumyantseva, V., Makhova, A., Bulko, T., Kuzikov, A., Shich, E., et al. (2015) Electrocatalytic cycle of P450 cytochromes: The protective and stimulating roles of antioxidants, *RSC Adv.*, **87**, 71306-71313.
15. Shumyantseva, V. V., Bulko, T. V., Suprun, E. V., Chalenko, Y. M., Vagin, M. Y., et al. (2011) Electrochemical investigations of cytochrome P450, *Biochim. Biophys. Acta Proteins Proteomics*, **1814**, 94-101.
16. McDonnell, B., Hearty, S., Leonard, P., and O'Kennedy, R. (2009) Cardiac biomarkers and the case for point-of-care testing, *Clin. Biochem.*, **42**, 549-561.
17. Marenzi, G., Giorgio, M., Trinei, M., Moltrasio, M., Ravagnani, P., et al. (2010) Circulating cytochrome c as potential biomarker of impaired reperfusion in ST-segment elevation acute myocardial infarction, *Am. J. Cardiol.*, **106**, 1443-1449.
18. Schweitzer-Stenner, R. (2018) Relating the multi-functionality of cytochrome c to membrane binding and structural conversion, *Biophys. Rev.*, **10**, 1151-1185.
19. Manickam, P., Kaushik, A., Karunakaran, C., and Bhansali, S. (2017) Recent advances in cytochrome c biosensing technologies, *Biosens. Bioelectron.*, **87**, 654-668.
20. Yin, J., and Miao, P. (2016) Apoptosis evaluation by electrochemical techniques, *Chem. Asian J.*, **11**, 632-641.
21. Hasanzadeh, M., Shadjou, N., de la Guardia, M. (2017) Early stage diagnosis of programmed cell death (apoptosis) using electroanalysis: nanomaterial and methods overview, *Trends Anal. Chem.*, **93**, 199-211.
22. Nikolaev, S., Lemmens, L., Koessler, T., Blouin, J.-L., and Nouspikel, T. (2018) Circulating tumoral DNA: preanalytical validation and quality control in a diagnostic laboratory, *Anal. Biochem.*, **542**, 34-39.
23. Huffnagle, I. M., Joyner, A., Rumble, B., Hysa, S., Rudel, D., and Hvastkovs, E. G. (2014) Dual electrochemical and physiological apoptosis assay detection of *in vivo* generated nickel chloride induced DNA damage in *Caenorhabditis elegans*, *Anal. Chem.*, **86**, 8418-8424.
24. Sanjuán, I., Martin-Gómez, A. N., Graham, J., Hernández-Ibáñez, N., Banks, C., et al. (2018) The electrochemistry of 5-halocytosines at carbon based electrodes towards epigenetic sensing, *Electrochim. Acta*, **282**, 459-468.
25. Campos-Carrillo, A., Weitzel, J. N., Sahoo, P., Rockne, R., Mokhnatkin, J. V., et al. (2020) Circulating tumor DNA as an early cancer detection tool, *Pharmacol. Ther.*, **207**, 107-458.
26. Kogikoski, S., Jr., Paschoalino, W. J., Cantelli, L., Silva, W., and Kubota, L. T. (2019) Electrochemical sensing based on DNA nanotechnology, *Trends Anal. Chem.*, **118**, 597-605.
27. Zhang, Y., Zhang, W. B., Liu, C., Zhang, P., Balaeff, A., and Beratan, D. N. (2016) DNA charge transport: moving beyond 1D, *Surf. Sci.*, **652**, 33-38.
28. Machera, H. C., García-Fernándeza, N., Adsuar-Gómez, A., Porras-Lópezc, M., González-Calleb, A., et al. (2019) Donor-specific circulating cell free DNA as a non-invasive biomarker of graft injury in heart transplantation, *Clin. Chim. Acta*, **495**, 590-597.
29. Udomsinprasert, W., Poovorawan, Y., Chongsrisawat, V., Vejchapipat, P., Jittikoon, J., and Honsawek, S. (2019) Leukocyte mitochondrial DNA copy number as a potential

biomarker indicating poor outcome in biliary atresia and its association with oxidative DNA damage and telomere length, *Mitochondrion*, **47**, 1-9.
30. Shoja, Y., Kermanpur, A., and Karimzadeh, F. (2018) Diagnosis of EGFR exon21 L858R point mutation as lung cancer biomarker by electrochemical DNA biosensor based on reduced graphene oxide/functionalized ordered mesoporous carbon/Ni-oxyte-tracycline metallopolymer nanoparticles modified pencil graphite electrode, *Biosens. Bioelectron.*, **113**, 108-115.
31. Brotons, A., Vidal-Iglesias, F., Solla, J., and Iniesta, J. (2016) Carbon materials for the electrooxidation of nucleobases, nucleosides and nucleotides toward cytosine methylation detection: a review, *Anal. Methods*, **8**, 702-715.
32. Herl, T., Taraba, L., Bohm, D., and Marysik, F.-M. (2019) Electrooxidation of cytosine on bare screen-printed carbon electrodes studied by on-line electrochemistry-capillary electrophoresis-mass spectrometry, *Electrochem. Commun.*, **99**, 41-45.
33. Li, C.-C., Wang, Z.-Y., Wang, L.-J., and Zhang, C.-Y. (2019) Biosensors for epigenetic biomarkers detection: a review, *Biosens. Bioelectron.*, **144**, 111-695.
34. Wang, G., Liu, Y., and Hu, N. (2007) Comparative electrochemical study of myoglobin loaded in different types of layer-by-layer assembly films, *Electrochim. Acta*, **53**, 2071-2079.
35. Shumyantseva, V. V., Sigolaeva, L. V., Agafonova, L. E., Bulko, T. V., Pergushov, D. V., et al. (2015) Facilitated biosensing via direct electron transfer of myoglobin integrated into diblock copolymer/multi-walled carbon nanotube nanocomposites, *J. Mater. Chem. B*, **3**, 5467-5477.
36. Alim, S., Vejayan, J., Yusoff, M., and Kafi, A. K. M. (2018) Recent uses of carbon nanotubes and gold nanoparticles in electrochemistry with application in biosensing, *Biosens. Bioelectron.*, **121**, 125-136.
37. Matveeva, E. G., Gryczynski, Z., and Lakowicz, J. R. (2005) Myoglobin immunoassay based on metal particle-enhanced fluorescence, *J. Immunol. Methods*, **302**, 26-35.
38. Tang, Z., Huang, J., He, H., Ma, C., and Wang, K. (2020) Contributing to liquid biopsy: Optical and electrochemical methods in cancer biomarker analysis, *Coord. Chem. Rev.*, **415**, 213-317.
39. Li, Q., Batchelor-McAuley, C., and Compon, R. G. (2010) Electrochemical oxidation of guanine: electrode reaction mechanism and tailoring carbon electrode surface to switch between adsorptive and diffusional responses, *J. Phys. Chem. B*, **114**, 7423-7428.
40. Gonзalves, L. M., Bachelor-McAuley, C., Barros, A., and Comton, R. G. (2010) Electrochemical oxidation of adenine: a mixed adsorption and diffusion response on an edge-plane pyrolytic graphite electrode, *J. Phys. Chem. C*, **114**, 14213-14219.
41. Trotter, M., Borst, N., Thewes, R., and von Stetten, F. (2020) Review: electrochemical DNA sensing – principles, commercial systems, and applications, *Biosens. Bioelectron.*, **154**, 112069.
42. Blair, E., Damion, K., and Corrigan, D. R. (2019) A review of microfabricated electrochemical biosensors for DNA detection, *Biosens. Bioelectron.*, **134**, 57-67.
43. Arvand, M., Niazi, A., Mazhabi, R. M., and Biparva, P. (2012) Direct electrochemistry of adenine on multiwalled carbon nanotube-ionic liquid composite film modified carbon paste electrode and its determination in DNA, *J. Mol. Liquids*, **173**, 1-7.
44. Sun, W., Li, Y., Duan, Y., and Jiao, K. (2008) Direct electrocatalytic oxidation of adenine and guanine on carbon ionic liquid electrode and the simultaneous determination, *Biosens. Bioelectron.*, **24**, 988-993.
45. Hasoň, S., Fojta, M., and Ostatná, V. (2019) Label-free electrochemical analysis of purine nucleotides and nucleobaes at disposable carbon electrodes in microliter volumes, *J. Electroanal. Chem.*, **847**, 113-252.
46. Palecek, E., and Bartosik, M. (2012) Electrochemistry of nucleic acids, *Chem. Rev.*, **112**, 3427-3481.
47. Reipa, V., Atha, D. H., Coskun, S. H., Sims, C. M., and Nelson, B. C. (2018) Controlled potential electro-oxidation of genomic DNA, *PLoS One*, **13**, e0190907.
48. Kowalczyk, A. (2020) Trends and perspectives in DNA biosensors as diagnostic devices, *Curr. Opin. Electrochem.*, **23**, 36-41.
49. Ji, L., Yu, S., Zhou, X., Bao, Y., Yang, F., et al. (2019) Modification of electron structure on the semiconducting single-walled carbon nanotubes for effectively electrosensing guanine and adenine, *Anal. Chim. Acta*, **1079**, 86-93.
50. Zhang, S., Zhuang, X., Chen, D., Luan, F., He, T., et al. (2019) Simultaneous voltammetric determination of guanine and adenine using MnO2 nanosheets and ionic liquid-functionalized graphene combined with a permeation-selective polydopamine membrane, *Microchim. Acta*, **186**, 450.
51. Ren, S., Wang, H., Zhang, H., Yu, L., Li, M., and Li, M. (2015) Direct electrocatalytic and simultaneous determination of purine and pyrimidine DNA bases using novel mesoporous carbon fibers as electrocatalyst, *J. Electroanal. Chem.*, **750**, 65-73.
52. Wang, H., Ma, R., Sun, F., Jia, L., Zhang, W., et al. (2018) A versatile label-free electrochemical biosensor for circulating tumor DNA based on dual enzyme assisted multiple amplification strategy, *Biosens. Bioelectron.*, **122**, 224-230.
53. Sigolaeva, L. V., Bulko, T. V., Kozin, M. S., Zhang, W., Köhler, M., et al. (2019) Long-term stable poly(ionic liquid)/MWCNTs inks enable enhanced surface modification for electrooxidative detection and quantification of dsDNA, *Polymer*, **168**, 95-103.
54. Shumyantseva, V. V., Agafonova, L. E., Bulko, T. V., Kuzikov, A. V., and Masamrekh, R. A. (2020) Preparation of electrochemical biosensor systems for the analysis of bioobjects: reasonable choice of working surface modifications for studies in the "smart-electrode" mode, *Biomed. Chem. Res. Methods*, **3**, e00119.
55. Shumyantseva, V. V., Bulko, T. V., Kuzikov, A. V., Masamrekh, R. A., Konyakhina, A. Yu., et al. (2020) All-electrochemical nanocomposite two-electrode setup for quantification of drugs and study their electrocatalytical conversion by cytochromes P450, *Electrochim. Acta*, **336**, 135579.
56. Sigolaeva, L. V., Bulko, T. V., Konyakhina, A. Yu., Kuzikov, A. V., Masamrekh, R. A., et al. (2020) Rational design of amphiphilic diblock copolymer/MWCNT surface modifiers and their application for direct electrochemical sensing of DNA, *Polymers*, **12**, 1514.
57. Aghamiri, Z. S., Mohsennia, M., and Rafiee-Pour, H.-A. (2018) Immobilization of cytochrome c and its application as electrochemical biosensors, *Talanta*, **176**, 195-207.

58. Santucci, R., Sinibaldi, F., Cozza, P., Polticelli, F., and Fiorucci, L. (2019) Cytochrome *c*: an extreme multifunctional protein with a key role in cell fate, *Int. J. Biol. Macromol.*, **136**, 1237-1246.
59. Shumyantseva, V. V., Bulko, T. V., Kuzikov, A. V., Masamrekh, R. A., Pergushov, D. V., et al. (2020) Electrochemical fingerprint of cytochrome *c* on a MWCNT/polymer nanocomposite electrode, *Mendeleev Commun.*, **30**, 299-301.
60. Lin, Y.-W. (2018) Structure and function of heme proteins regulated by diverse post-translational modifications, *Arch. Biochem. Biophys.*, **641**, 1-30.
61. Rodriguez-Roldan, V., Garcia-Heredia, J., Navarro, J., De la Rosa, M., and Hervas, M. (2008) Effect of nitration on the physicochemical and kinetic features of wild-type and monotyrosine mutants of human respiratory cytochrome *c*, *Biochemistry*, **47**, 12371-12379.
62. Ly, H. K., Utesch, T., Diaz-Moreno, I, Garcia-Heredia, J. M., De La Rosa, M. A., and Hildebrandt, P. (2012) Perturbation of the redox site structure of cytochrome *c* variants upon tyrosine nitration, *J. Phys. Chem. B*, **116**, 5694-5702.
63. Lee, I., Salomon, A., Yu, K., Doan, J. W., Grossman, L., and Huttemann, M. (2006) New prospects for an old enzyme: mammalian cytochrome *c* is tyrosine-phosphorylated *in vivo*, *Biochemistry*, **45**, 9121-9128.
64. Yu, H., Lee, I., Salomon, K., Hüttemann, Yu. M. (2008) Mammalian liver cytochrome *c* is tyrosine-48 phosphorylated *in vivo*, inhibiting mitochondrial respiration, *Biochim. Biophys. Acta*, **1777**, 1066-1071.
65. Kamel, M., Raissi, H., Hashemzadeh, H., and Mohammadifard, K. (2020) Theoretical elucidation of the amino acid interaction with graphene and functionalized graphene nanosheets: insights from DFT calculation and MD simulation, *Amino Acids*, **52**, 1465-1478.